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Full Length Research Paper

Good hygiene practices and microbiological contamination in commercial restaurants

Rosiane Cosme Nascimento, Elizandra Maier Silva and Jackline Freitas
Brilhante de São José*

Department of Integrated Education in Health, Federal University of Espírito Santo, Marechal Campos Avenue - 1468, Campus Maruípe, Vitória, CEP 29043-900 - Espírito Santo, Brazil.

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Food services and commercial restaurants have gained more space in people's lives. Thus, people are more concerned about food security and sanitary hygiene. The present study evaluated practices of manipulation and contamination of environments and the preparation of surfaces in commercial restaurants in Vitória-ES, Brazil. Data on good practices were collected through a checklist proposal based on the RDC 216/2004; air samples were collected via the simple sedimentation technique, while surfaces (countertops and utensils) were sampled via the swab technique to analyze the presence of microbial indicators (n = 12). Regarding buildings, facilities and utensils block, 50% of the restaurants were classified as unsatisfactory. In food handlers and storage and transport of prepared food blocks, 58 and 100% of the restaurants, respectively, were classified as unsatisfactory. 83% of the restaurants being classified as unsatisfactory in documentation and registration block, with emphasis on the lack of or inadequacy of the Good Practice Manual. The hygienic and sanitary conditions were considered unsatisfactory in most restaurants evaluated, representing a low agreement with the legislation. Air contamination levels were above the recommendations, which indicates inadequate practices in some of the establishments. All establishments were unsatisfactory when mesophilic bacteria were analyzed on countertops surfaces. Values of the order of 10^5 of aerobic mesophiles for knives were observed. These results, together with the high percentage of inadequations, indicate the need for immediate action for control and prevention as well as a greater supervision by the competent organizations. Actions to reduce the risks of contamination and to ensure greater consumer safety are crucial.

Key words: Food quality, food service, quality control.

INTRODUCTION

With urbanization, extended working hours and a greater insertion of women into the labor market, the number of meals prepared out of the home has increased. As a consequence, food services, such as

those offered in commercial restaurants, have gained more space in people's lives. This change in behavior has led to a greater concern about the food offered, especially from the point of view of hygienic and

*Corresponding author. E-mail: jackline.jose@ufes.br.

sanitary quality assurance (Ferreira et al., 2009; Coelho et al., 2010). The main causes of food contamination are associated with the development of undesirable microorganisms, which may render food repulsive due to the deterioration or the health risks posed by the presence of pathogenic bacteria. Transmission can occur through manipulation, inadequate preservation and or environmental contamination, since most pathogenic microorganisms are not normally present in food (Ferreira et al., 2009; Di Ciccio et al., 2015).

Foodborne diseases (FBD) are diseases caused by the presence of pathogenic microorganisms in food. These diseases are one of the main consequences of the lack of hygienic and sanitary control in the collective feeding sector, where biological, physical, and chemical hazards are found (Rahman et al., 2016). According to Rahman et al. (2016), the Centers for Disease Control and Prevention (CDC) have reported numerous different foodborne pathogens that can cause infections in humans.

To ensure safety in food production, it is necessary to implement good handling practices and standard operating procedures, which are a set of rules for the correct handling of food from the raw material to the final product and define when, why, how, and where activities should be carried out, thereby indicating the records used to ensure compliance with operations (Djekic et al., 2016).

In conjunction with good practices, the assessment of the microbiological conditions of food preparation sectors becomes essential for the production of quality meals. Poor equipment and utensil hygiene has been responsible, alone or associated with other factors, for outbreaks of FBD or for alterations in processed foods (Doménech-Sánchez et al., 2011).

Surfaces used for food preparation, such as appliances or utensils, may appear to be clean, but this condition may be misleading. If the preparation surface remains moist and has food residues, it may allow the adhesion of microorganisms and possibly the formation of microbial biofilms, which makes the cleaning process more difficult and increases the risks of cross-contamination (Andrade, 2008). Food contamination can occur in different stages of the food production process, from the receipt of the raw materials to the distribution of the preparations. Exposure of pathogens to surfaces can occur by direct contact with contaminated materials or indirectly through microbiota in the air (Di Ciccio et al., 2015).

In addition to the evaluation of the preparation areas, it is important to monitor the microbiological contamination of the air, which is characterized by aerosols formed by the vegetative cells of bacteria deposited on dust particles (Andrade, 2008). In food processing/preparation areas, routine employee activities, floor drains, ventilation systems, communication between different sectors, and equipment surfaces

are recognized sources of aerosols (Byrne et al., 2008; Coelho et al., 2010).

The microorganisms present in these aerosols can travel by air and reach the food during preparation stages (São José, 2012). In view of the above, the objective of this study was to evaluate the adequacy of good handling practices and the microbiological contamination of environments and preparation surfaces in commercial restaurants in Vitória, Espírito Santo, Brazil.

MATERIALS AND METHODS

A cross-sectional study was conducted in commercial restaurants, located in Vitória-ES, between August and September 2015. For sample, locations close to the institution were preferred and then the microbiological analyses were carried out, totaling 12 self-service restaurants. The establishments were contacted by means of an invitation letter to present the research objectives, and then permission to visit was requested. All persons responsible for the participating establishments signed an authorization term guaranteeing the research (application of checklist and collection of air samples and surfaces). The analyses were conducted in the Laboratory of Microbiology of the Department of Pharmaceutical Sciences - Health Sciences Center, Federal University of Espírito Santo.

Assessment of good practices

Data collection on good manipulation practices was done through direct observation during visits by a trained researcher. For the evaluation, a checklist was proposed based on Resolution RDC 216/2004 (Brazil, 2004), divided into three parts: company identification, evaluation, and classification of the establishment. The checklist presented 12 question blocks evaluated in each restaurant, totaling 91 items, as follows: 'buildings, facilities, furniture and utensils' (17 items); 'Hygiene of facilities, equipment, furniture and utensils' (nine items); 'Integrated control of vector and pest' (three items); 'Water supply' (four items); 'Waste management' (three items); 'food handlers' (nine items); 'Food, ingredients, and packaging' (six items); 'Food preparation' (20 items); 'Storage and transport of prepared food' (three items); 'Exposure to the consumption of prepared food' (seven items); 'Documentation and registration' (eight items); 'Responsibility' (two items). Each item had three possible answers: 'Conform', 'Not Conform', and 'Not applicable' (NA). Subsequently, the classification was based on the scoring criteria established in item D of RDC 275/2002 (Brazil, 2002), namely: Good (76 to 100% attendance of items), Regular (51 to 75% attendance of items), and Unsatisfactory (0 to 50% of attendance of the items).

Microbiological analyses

Microbiological analyses consisted of the evaluation of air contamination and food preparation surfaces. Air sampling was carried out using the simple sedimentation technique in Petri dishes containing appropriate culture media for each microbial group evaluated, according to the methodology proposed by Evancho et al. (2001). Aerobic mesophile microorganism counts were performed on plates containing standard agar for counting (Acumedia®) after incubation for 24 to 48 h at 37°C. For counting of molds and yeasts, potato dextrose agar (Acumedia®), acidified with 1.5 mL of 10% tartaric acid for each 100 mL of medium, was

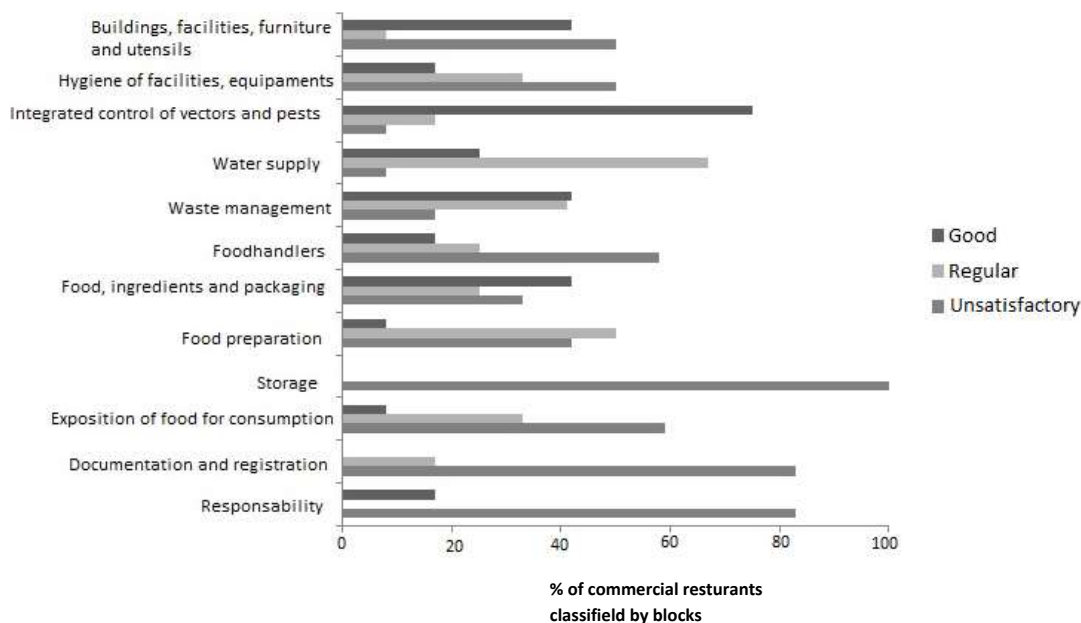


Figure 1. Classification by blocks regarding hygienic and sanitary conditions of commercial restaurants in Vitória-ES, Brazil, 2015.

used for incubation at 25°C for five to seven days. Gram-negative enteric bacteria were observed in MacConkey agar medium (Acumedia®), and the plates were incubated at 37°C for 48 h. The results, expressed as colony-forming units/cm² /week (CFU/cm²/week), were calculated according to the following formula (Andrade, 2008):

$$\text{Viable particles for cm}^2/\text{week} = (\text{CFU } 10,080^*) / [(\pi r^2) t],$$

where r = the radius of the Petri dish, in cm; π = 3.141516; T = time of exposure of Petri dishes (minutes); and $*$ = minutes for a week.

For the analysis of the microbiological conditions of the preparation surfaces (benches and utensils), the swab technique was applied according to the American Public Health Association (APHA), as described by Evancho et al. (2001). On each bench surface (pre-preparation of meats and vegetables) and board, we collected samples at two 25-cm² points, using a previously sterilized mold; sampling was performed after the routine cleaning of the establishments. For the surfaces of utensils (knives), collection was performed on any area that comes into contact with food. Utensil samples were collected for each establishment, sampling a knife and a board, the most used utensils in the pre-preparation process. The selection of these utensils for the collection of the samples was based to the high risk of cross contamination that can occur with the use of inappropriate materials. The samples were transported to the laboratory in isothermal boxes immediately after collection; decimal dilutions were prepared for sowing in Petri dishes for incubation and subsequent counting.

Statistical analyses

The data obtained in the evaluation of hygienic and sanitary conditions through the checklist were entered into Microsoft Excel® and analyzed for the percentage of items matching by

blocks. For the analysis of the results in regard to the contaminating microbiota in the air and on the preparation surfaces of the restaurants, an evaluation was made regarding the compliance with the proposed recommendations APHA as described by Evancho et al. (2001). Descriptive analysis of the data was performed, presenting means, percentages of the counts and their standard deviations.

RESULTS AND DISCUSSION

Adequacy of commercial restaurants in terms of good block manipulation practices

Figure 1 shows the classification by blocks of hygienic and sanitary conditions of the commercial restaurants evaluated. This classification is based on the 12 aspects included in the checklist of good practices, which enabled a more detailed assessment of the main inadequacies observed.

Regarding buildings, facilities, furniture, and utensils, 42% of the restaurants were classified as good and 50% as unsatisfactory. Among the irregularities identified, the following stand out: lack of ordering in the flow of operations; access of the common food area to other areas of the establishment; doors and windows without barriers (rubber sealing, millimeter screens); exposed luminaires without protection; air flow directly over food; floors, walls, and ceilings with cracks, leaks, and infiltrations; paper towel shortage, trash cans without covers or with manual operation and absence of an exclusive lavatory for hand hygiene in the handling area. All inadequacies indicate critical conditions for the production of meals. Similar results were found in the

study by Genta et al. (2005), where nonconformity index values ranged from 12.5 to 53.1% in commercial restaurants.

In the block referring to the hygiene of facilities, equipment, furniture, and utensils, 50% of establishments were classified as unsatisfactory. The main inadequacies observed were as follows: poor conditions of conservation and hygiene of equipment; lack of training of the employees to carry out the hygiene operations and the maintenance of them; use of common uniforms for handling food and for cleaning the environment and facilities; sanitizing products not regulated by the Ministry of Health. Rossi (2006) also found unsatisfactory results for this block and observed less than 50% of adequacy in self-service commercial restaurants.

Regarding vector and pest control, 75% of the restaurants were classified as good, while only 8% of commercial establishments were classified as unsatisfactory. A set of preventive actions was observed, such as chemical control carried out by a specialized company, according to the specific legislation. According to RDC 216/2004 (Brazil, 2004), buildings, facilities, equipment, furniture, and utensils must be free of vectors and urban pests, and in the case of chemical control, they must be sanitized later to remove any residues. Germano et al. (2001) stress that the presence of animals may be related to deficient structures and a lack of knowledge about preventive, corrective, and control programs.

In terms of water supply, adequate conditions were diagnosed in only 25% of the establishments and 67% were classified as regular. Water is used in the preparation of food and in the hygiene of the contact surfaces of these products; good water quality is therefore of utmost importance. Water quality control is necessary to avoid possible health risks for consumers in this type of environment (Andrade, 2008).

Considering waste management, the percentage of adequacy in restaurants was 42% (good), while only 17% were inadequate (unsatisfactory). However, we observed that residues were deposited in inappropriate places, in some establishments near the entrance of the dining rooms. Garbage is a source of food contamination, as it favors the appearance of vectors and urban pests. According to RDC 216/2004, waste must be frequently collected and stored indoors, away from the food preparation area (Brazil, 2004).

Regarding food handlers, only 17% of the restaurants were classified as good and 58% as unsatisfactory. Handlers did not sanitize hands carefully when they arrived at work, either before or after handling food. In addition, a lack of orientation posters on the correct washing and antisepsis procedures of the hands and improper behavior of the food handlers who spoke, whistled, and coughed during the preparation of meals were noted. There were no records of periodic training

of employees on topics such as personal hygiene, foodborne diseases, and food hygiene. In a study by Genta et al (2005), this block presented between 12.5 and 56.3% of inadequacy.

Various studies confirmed the presence of pathogenic microorganisms in food handlers' hands, which makes them a significant vehicle of foodborne diseases (Soares, et al., 2012; Ferreira et al., 2013). According to legislation (Brazil, 2004), the health state of the food handlers and the hygienic practices directly influence the hygienic and sanitary safety of foods, with the majority of cases of food infections and intoxications occurring due to the contamination of food by food handlers.

For the raw materials, ingredients, and packaging block, 42 and 25% were classified as good and fair, respectively. The main inadequacies were related to the place of the reception of raw material, which was connected to the distribution hall; shelves that did not exhibit the minimum spacing necessary to guarantee adequate ventilation, cleaning and disinfection of the place were inadequate, and food packaging was inappropriate. According to the guidelines, all material used for the packaging of ingredients and raw materials must effectively avoid contamination and must not subject food to undesirable substances that exceed the limits proposed by the competent bodies (Brazil, 2004).

In the food preparation block, only one restaurant (8%) was adequate, while 42% were classified as unsatisfactory. In these establishments, time and temperature control in the preparations were not checked, food was defrosted incorrectly, the ingredients had no labels with expiration dates, and the quantities of employees, equipment, furniture, and/or utensils were not compatible with the complexity of food preparations. Rossi (2006) identified that few restaurants met these requirements in terms of temperature and time control procedures; 10% of the 30 establishments analyzed had a preventive maintenance and equipment calibration program, and in only 13.3% of the restaurants, there were spreadsheets for temperature recording.

In terms of storage and transport of the prepared food, 100% of the restaurants were classified as unsatisfactory; this is extremely worrying, as this stage confers significant risks of contamination and microbial multiplication. In this block, we observed the following inadequacies: a lack of identification of prepared foods that are kept in the storage area or awaiting transportation, not conferring protection against contaminants; no monitoring of the time and temperature during the storage, transport, and distribution stages; the vehicles also carried other loads, thus compromising the quality of the food. It is extremely important to control storage time and temperature as well as the sanitary conditions of foods already prepared in order to avoid microbial

Table 1. Microbiological contamination of air in commercial restaurants, Vitória-ES, Brazil, 2015.

Surface	Count intervals (CFU/cm ² /week)		
	Aerobic mesophiles	Molds and yeasts	Enterobacteria
Meat preparation sector*	ND - 5.0 x 10 ²	ND - 8.5 x 10 ²	ND - 3.6 x 10 ²
Fruit and vegetable preparation sector*	1.3 x 10 ² -9.8 x 10 ²	ND - 1.1 x 10 ²	ND - 8.6 x 10 ²

ND= Not detected; *Simple sedimentation method.

contamination. In many cases, this is a critical control point, since there will be no stages to eliminate or minimize the presence of contaminants.

Regarding the consumption of prepared food, only 8% of the restaurants were classified as adequate (Good), while 59% were classified as unsatisfactory. In the latter, the prepared food was not controlled for temperature. The monitoring of time and temperature is essential to avoid microbial growth and the contamination of prepared foods. In addition, equipment and utensils were in poor condition; equipment exposed to food had no protective barriers; areas of exposure and preparation of disorganized food and without appropriate hygienic and sanitary conditions; money and food were handled by the same employee (Brazil, 2004).

For the documentation and registration questionnaire, the results presented a percentage of worrisome inadequacies, with 83% of the restaurants being classified as unsatisfactory, with emphasis on the lack of or inadequacy of the Good Practice Manual (GPM) and the Standard Operating Procedures (SOP). The results obtained by Genta et al. (2005) corroborate with our findings; the authors observed that none of the evaluated establishments had a GPM. It is worth mentioning that those responsible for the establishments evaluated could not produce training courses on food handling. Souza et al (2013) verified that after the implementation of GPM in a food and nutrition unit, significant changes occurred in the establishment, which were even more expressive after the training of the employees, who started to place more emphasis on hygiene. Finally, in terms of liability, the lack of training of personnel, from the technical leaders to the food handlers, stands out.

Evaluation of the microbiological contamination of air

Table 1 shows ranges of counts for aerobic mesophiles, molds, yeasts, and enterobacteria present in air samples of environments in commercial restaurants evaluated. When adopting the limit of 30 CFU/cm²/week for aerobic mesophiles stipulated by the APHA (Evancho et al., 2001), the levels were above the

recommendations, which indicates inadequate practices in some of the establishments.

For the evaluated restaurants, the sectors associated to the pre-preparation of vegetables presented the highest air contamination with aerobic mesophiles and enterobacteria, with the values of 9.8 x 10² and 8.6 x 10² CFU/cm²/week, respectively. Coelho et al. (2010) identified a mesophilic count of 10³ CFU/cm²/week in commercial restaurants in the beef pre-processing sector, which is 30 times higher than that recommended by the APHA (Evancho et al., 2001). Our results were similar to those found by Tomich et al. (2005), who reported that 85.7% of the samples collected from a food industry presented scores above the limits proposed by the American legislation. However, the absence of specific recommendations in Brazil for each group of microorganisms makes it difficult to evaluate the results obtained.

For molds and yeasts, the air in the pre-prepared meat sector also presented a higher contamination, with 8.5 x 10² CFU/cm²/week, in relation to the other microorganisms analyzed. The facts that there were no on-site partitions and that the kitchen and the external environment (bathrooms, dining rooms, reception, and customer service) were connected through doors without automatic closing and windows without screens may have favored the observed high contamination. Air filtering and other quality control measures are essential, such as air distribution in the processing areas and evaluation of the layout of the establishment (São José, 2012).

Evaluation of the contamination of food preparation surfaces

High counts of aerobic mesophiles were observed on benches and utensils (Table 2). Values of the order of 10⁵ for knives were observed, and when considering the APHA (Evancho et al., 2001) recommendations, counts of up to 2 CFU/cm² for countertop surfaces and 100 CFU/utensil were found; a large part of the surfaces were in unsatisfactory conditions.

In terms of mesophiles on bench surfaces, 100% of the establishments were unsatisfactory, while for

Table 2. Microbiological contamination of bench and utensil surfaces in commercial restaurants in the city of Vitória-ES, 2015.

Surface	Count intervals (CFU/cm ² ou CFU/utensil)		
	Mesophile aerobics	Molds and yeasts	Enterobacteria
Meat preparation bench*	$2.1 \times 10^4 - 1.1 \times 10^6$	ND - 3.2×10^4	ND - 3.9×10^5
Fruit and vegetable preparation bench *	$4.0 \times 10^2 - 1.2 \times 10^6$	$3.0 \times 10^1 - 9.3 \times 10^3$	ND - 3.0×10^5
Knife*	ND - 7.6×10^5	ND - 1.2×10^4	ND - 3.5×10^5
Cutting board*	ND - 1.1×10^6	ND - 5.6×10^4	ND - 5.3×10^5

ND= Not detected; *Simple sedimentation method.

mesophiles on utensils, 79.1% were inadequate. It is worth noting that there are productive areas that are more prone to surface contamination, such as the pre-processing of fruits, vegetables, and raw meats (Lahou et al., 2012). This is mainly because food arriving in these sectors has already been contaminated in the production places, and the microorganisms can accumulate due to inappropriate hygiene techniques.

Similar results were found by Coelho et al. (2010), where 71% of the equipment and utensils showed values above those established by the APHA (Evancho et al., 2001). In a study by Andrade et al. (2003), 18.6% of the equipment and utensils used in the preparation of food had aerobic mesophile counts of up to 2 CFU/cm² of surface. The APHA recommendation (Evancho et al., 2001) is considered very rigid for Brazilian restaurants, taking into account, in particular, ambient temperature conditions in Brazil that are quite different from those in America.

The highest counts were found on boards used for the preparation of meats and vegetables, suggesting that there is no adequate application of good handling practices and SOP. Poor hygiene of equipment and utensils has been responsible, alone or associated with other factors, for outbreaks of foodborne illness (Doménech-Sánchez et al., 2011). All surfaces in contact with food may exhibit residues of organic matter, which may influence the survival of food-borne pathogens in this environment (Djekic et al., 2016). In addition, when assembling the food preparation environment, with benches, equipment, and utensils, the technician should be aware of the appropriate choice of materials. In contrast to aerobic mesophiles, counts for molds and yeasts (Table 2) varied between not detected (ND) up to 10⁴ CFU were, which may be related to the measures adopted for the control of vectors and urban pests and circulating air currents in the food preparation environment (Brazil, 2004).

Boards had the highest levels of molds and yeast counts, reaching 5.6×10^4 CFU/cm², indicating the possibility of cross contamination because of the lack of routine sanitization. Similar results were found by Battaglini et al. (2012), where cutting boards were the surfaces that presented the highest mean yeast counts (2.1×10^4 and 1.5×10^4 CFU/cm²).

Rodríguez et al. (2011), when evaluating handling practices and microbiological conditions of ready-to-eat products, verified that the highest contaminations were recorded on cutting board surfaces and taps. Cutting boards represent a constant risk of contamination in restaurants and the domestic environment. Although they are popular in both environments, there may be significant cross-contamination risks when using these utensils (DeVere and Purchase, 2007).

In the group of enterobacteria, tables presented the most unsatisfactory values, while the lowest counts were observed on knives. These microorganisms, of which some are pathogenic, are involved in food deterioration processes and used as indicators of hygienic and sanitary quality. Enterobacteria may indicate inadequate sanitary conditions when present in food or on surfaces (Silva Jr, 2014; Souza et al., 2015).

The type of material used in the analyzed surfaces may have contributed to the results found in this study. A number of countertops in the meat and vegetable preparation sector were made of marble, while the handling boards were made of allyl and unfit for use. Regarding the knives, all had a stainless-steel cutting surface; however, in some establishments, they were inadequate because of signs of corrosion. Food preparation equipment and utensils must be made of special materials to prevent them from absorbing or releasing particles that may interfere with the composition of foodstuffs; they must be kept in a good state of preservation.

The presence of microorganisms on food preparation surfaces indicates that cleaning is not efficient, resulting in considerable risks. Food preparation surfaces must be smooth, hard, and without cracks, ensuring lower microbial adhesion. These characteristics ensure adequate hygiene procedures (Andrade, 2008; São José, 2012; Sol et al., 2018).

The hygienic and sanitary conditions were considered unsatisfactory in most commercial restaurants evaluated, representing a low compliance with the criteria required by the legislation. In this way, the importance of preventive actions and measures that can reduce the risks of contamination and guarantee greater security is highlighted. Food safety is essential in food service because of the high numbers of meals served

day by day (Rebouças et al., 2017).

Nutritionists are responsible for complying with and enforcing the health surveillance legislation, which in turn include sanitary surveillance, promotion, and participation in educational activities in this area. The role of this professional is indispensable to correct the flaws and nonconformities in the establishments and to enforce legislation.

Conclusion

Based on the results obtained, there is a clear need for adjustments in the analyzed restaurants as well as for the implementation of Good Practices and a greater oversight of the competent bodies, since most restaurants did not comply with the RDC 216/2004, mainly in terms of food storage, documentation, registration, and responsibilities. The inadequacies detected in the evaluation of good practices may have resulted in the high levels of contamination observed for both the preparation surfaces and the ambient air. This shows the need for regulated microbiological standards in Brazil to evaluate the quality of air and surfaces in the food production environment.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

***blaKPC-2* and *blaOXA-48* producing *Klebsiella pneumoniae* found in a Turkish hospital in the Balkans**Sebnem Bukavaz^{1*}, Metin Budak² and Aygül Dogan Celik³¹Department of Medical Microbiology, Faculty of Medicine, Trakya University, Edirne, Turkey.²Department of Biophysics, Faculty of Medicine, Trakya University, Edirne, Turkey.³VM Medical Park Hospital, Kocaeli, Turkey.

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Carbapenem-resistant *Klebsiella pneumoniae* carrying *blaKPC-2*, *blaOxa48*, and other metallo-β-lactamases (MBLs) are difficult to treat. This study was conducted to study the phenotypic and genotypic features of carbapenem resistance in isolates of *K. pneumoniae* isolated from a tertiary care hospital in the European region of Turkey. Isolates of *K. pneumoniae* (n=100) resistant to at least one carbapenem (imipenem, meropenem, or ertapenem) were collected from 60 patients for 14 months. Carbapenem resistance was determined via the VITEK-2 system and the E-test confirmed this. The Modified Hodge Test (MHT) and Double Disc Synergy Test (DDST) were performed. Genes were analyzed by Sybr-Green real-time, multiplex and conventional polymerase chain reaction (PCR). Phylogenetic relatedness was analyzed by ERIC-PCR. The rate of resistance by E-test against ertapenem, imipenem, and meropenem were 98, 94, and 72%, respectively; 21% of isolates were somewhat susceptible to tygecycline. The MHT positivity was 98% and DDST was negative in all cases. There were 19 and 24% of isolates positive for *blaKPC-2* and *blaOxa-48*, respectively. ERIC-PCR showed that all *blaKPC-2*-positive isolates were branched into two main clusters with 80.5% similarity. The results indicate that *blaKPC-2* mediated carbapenem-resistant *Klebsiella pneumoniae* (CRKP) infection is spreading in Turkey and *blaOXA-48* endemicity continues to be a serious problem. The molecular determination of carbapenemases will be useful for patients with concurrent carbapenem-resistant Enterobacteriaceae (CRE) infections. This could prevented outbreaks and complications.

Key words: *Klebsiella pneumoniae*, carbapenem resistance, *blaKPC-2*, *blaOxa-48*.

INTRODUCTION

Carbapenems, among the beta-lactams, are the most effective agents against Gram-positive and Gram-negative bacteria presenting an expansive antimicrobial spectrum. Their unique molecular structure is due to the

presence of a carbapenem together with the beta-lactam ring. This combination confers exceptional stability against most beta-lactamases (enzymes that inactivate beta-lactams) including ampicillin and carbenicillin (AmpC)

*Corresponding author. E-mail: sbukavaz@hotmail.com. Tel: 0090 542 571443.

and the extended spectrum beta-lactamases (ESBLs) (Meletis, 2016). The emergence and dissemination of carbapenem-resistant Gram-negative pathogens including *Pseudomonas aeruginosa*, *Acinetobacter baumannii* as well as Enterobacteriaceae and Gram positive bacteria (e.g., *Staphylococcus* and *Streptococcus* species) is a significant contributor to patient morbidity and mortality (Patel et al., 2013). The emergence and spread of carbapenem-resistant Enterobacteriaceae is a major public health challenge. The most prevalent carbapenemases in *Klebsiella pneumoniae* are *K. pneumoniae* carbapenemases (KPC) in Amber class A which was first identified in 1996 in the United States (Yigit et al., 2001) and it is now endemic to several non-contiguous areas of the world especially China, Israel, Greece, and South America (Munoz-Price et al., 2013; Li et al., 2016). KPC-producing *K. pneumoniae* has only recently been reported in Turkey (Labarca, 2014). The other prevalent enzyme is Oxacillinase (OXA-like) carbapenemase in class D; specially *blaOxa-48* type with strong carbapenem-hydrolyzing activity was identified first in Turkey (Poirel et al., 2004). Over the years, numerous *blaOxa-48* type carbapenemase-producing Enterobacteriaceae have been reported in Belgium (Cuzon et al., 2008), Morocco (Benouda et al., 2010), Israel (Goren et al., 2011), Tunisia (Saidani et al., 2012), United Kingdom (Thomas et al., 2013), Lebanon (Hammoudi et al., 2014), India (Srinivasan et al., 2015), Argentina (Pasteran et al., 2012) and Czech Republic (Skalova et al., 2017). The increased frequency of report shows that it has successfully spread and has even become endemic (Carrer et al., 2010; Nazik et al., 2014; Sahin et al., 2015). The main question still remains unclear; whether the carbapenemases spread within Enterobacteriaceae is chromosomal or whether horizontal transfer because the gene encoding this enzyme is located in the plasmid; for instance ST258 specific clone is associated carriage of *blaKPC-2* and *blaKPC-3* genes, is considered to be responsible for expansion of resistance (van Duina and Doi, 2017).

The aim of this study was to describe data collected for 14 months regarding the clinical *K. pneumoniae* isolates from intensive care unit (ICU) patients as well as their microbiologic and molecular features. The site was Trakya University Health Research and Application Center and State Hospital located in the Balkans.

MATERIALS AND METHODS

Sample collection and bacterial diagnosis

This study was performed at a 1100-bed tertiary care center. A total of 100 *K. pneumoniae* isolates were isolated from 60 patients. These patients were being treated at different wards between January 2013 and February 2014. The clinical samples consisted of blood, endotracheal aspirate, urine, tissue biopsy, central venous catheter, abscess fluids, or wound. An automated VITEK-2 (BioMerieux, Bruz, France) system was used to identify the isolates.

The isolates were stored in the CryoBilles (AES Laboratoire, France) medium tube at the -80°C until molecular analysis.

Antimicrobial drug susceptibility testing and phenotypic screening of carbapenemase production

Antimicrobial susceptibility testing was conducted by automated VITEK-2 susceptibility card AST-GN325 (BioMerieux, Bruz, France) containing imipenem and meropenem tests according to the manufacturer's instructions. The MIC calling range for meropenem and imipenem on the AST-GN28 card was ≤ 0.25 to ≥ 16 $\mu\text{g/ml}$ in doubling dilutions. The MIC results were categorized as susceptible, intermediate, or resistant based on 2014 CLSI breakpoints (susceptible, ≤ 1 $\mu\text{g/ml}$; intermediate, 2 $\mu\text{g/ml}$; resistant, ≥ 4 $\mu\text{g/ml}$) (Wayne, 2014).

All isolates were tested for MIC values of imipenem, meropenem, ertapenem, and tigecycline via the E-test strip (BioMerieux, Durham, NC, USA). The results were evaluated for CLSI breakpoints for imipenem, meropenem, and ertapenem and US Food and Drug Administration breakpoints were used to evaluate to determine susceptibility of tigecycline: susceptible, ≤ 2 $\mu\text{g/ml}$; intermediate, 4 $\mu\text{g/ml}$; resistant, ≥ 8 $\mu\text{g/ml}$ (US FDA, 2009).

The Double Disc Synergy Test (DDST) and Modified Hodge Test (MHT) were used to phenotypically screen metallo- β -lactamases (Ramana et al., 2013). An imipenem (10 μg) disc and indicator isolate *E. coli* ATCC 25922 were used for MHT.

Detection of carbapenemase genes

Three different types of PCR techniques were applied via published primers (Table SD, supplementary data). SYBR-Green real-time PCR coupled with Melt-Curve analysis for *blaKPC* and *blaNDM*.

The other β -lactamases *blaVIM-1*, *2*, *blaSIM*, *blaGIM*, and *blaSPM* were screened by multiplex PCR. Conventional single PCR for *blaOxa-48* and for ERIC-PCR (details in supplemental data) were conducted before phylogenetic program analyses.

The PCR products were sequenced at Med Santek (Istanbul, Turkey) using the same primers used in PCR reactions with ABI 3100 Capillary Electrophoresis sequence detection system (Applied Biosystem, CA).

RESULTS

During the study period, 100 carbapenem-resistant *K. pneumoniae* (CRKP) isolates (one is susceptible to all antibiotics; clinical negative control) were isolated from different clinical materials from 60 patients. The maximum of four isolates of identical species isolated from different clinical materials or at the different dates of isolation were included. The isolates were obtained from blood (36%), urine (22%), endotracheal aspirate (21%), intravenous catheter (IVC) (15%), abscess aspiration (3%), sputum (2%), and wound tissue (1%). Most (76%) patients were in the intensive care unit and the other clinics were the internal medicine clinics (oncology, hematology, physical therapy and rehabilitation) (12%), surgical clinics (7%), and the emergency unit (5%). Antibiotic susceptibility test results indicated that all isolates were resistant to ampicillin, amoxicillin-clavulanic acid, and piperacillin-tazobactam. Between 94 and 68%

Table 1. MIC values of the strains according to the VITEK-2 system.

Antibiotic	MIC values ($\mu\text{g/ml}$)		
Meropenem	≥ 8 (96%)	2 (3%)	≤ 0.5 (1%)
Ertapenem	≥ 8 (99%)	-	≤ 0.5 (1%)
Imipenem	≥ 8 (97%)	2 (2%)	≤ 0.5 (1%)
Ampicillin	≥ 32 (100%)	-	-
Ampicillin/Clavulanic acid	≥ 32 (99%)	-	≤ 2 (1%)
Piperacillin tazobactam	≥ 128 (99%)	-	≤ 4 (1%)
Cefuroxime-Cefuroxime/Axetil	$\geq 16-64$ (98%)	-	$\leq 1-4$ (2%)
Cefoxitin	32-64 (96%)	-	≤ 4 (4%)
Ceftazidime	$\geq 16-64$ (68%)	8 (13%)	≤ 4 (19%)
Ceftriaxone	32-64 (94%)	2 (1%)	≤ 1 (5%)
Cefepime	$\geq 16-64$ (68%)	4-8 (5%)	≤ 2 (27%)
Trimethoprim-sulfamethoxazole	≥ 320 (60%)	-	≤ 20 (40%)
Ciprofloxacin	≥ 4 (49%)	-	≤ 0.25 (51%)
Amikacin	≥ 64 (28%)	16 (6%)	$\leq 2-4$ (67%)
Gentamicin	≥ 16 (38%)	-	$\leq 1-4$ (62%)
Colistin	≥ 16 (3%)	-	$\leq 0.5-2$ (97%)

Table 2. Susceptibility of the strains for carbapenems and tigecycline according to E-test.

Antibiotic	MIC values ($\mu\text{g/ml}$)		
Ertapenem	≥ 12 -no zone (98%)	-	0.012 (2%)
Imipenem	≥ 4 -no zone (94%)	3 (4%)	0.24 (2%)
Meropenem	≥ 4 -no zone (72%)	1.5-3 (19%)	≤ 1 (9%)
Tigecycline	-	3-4 (21%)	0.019- ≤ 2 (79%)

were resistant to cefuroxime, cefuroxime/axetil, cefoxitin, and ceftazidime. Variable resistances were observed to ceftriaxone (94%), cefepime (68%), trimethoprim-sulfamethoxazole (60%), and ciprofloxacin (49%). Resistance rates to ertapenem, imipenem, and meropenem were 99, 97, and 96%, respectively. The MIC values for all antibiotics (based on VITEK-2) are shown in Table 1. The resistance rates for ertapenem, imipenem, and meropenem were determined by the E-test to be 98, 94, and 72%, respectively. None of the isolates were resistant to tigecycline; 21% had intermediate resistant. The E-test results are shown in Table 2. The MHT result was positive for 98% of the isolates, but the DDST was negative for all.

Molecular analysis revealed that 19 and 24 isolates were positive for *blaKPC-2* and *blaOXA-48* genes, respectively. The *blaKPC-2* positive isolates were isolated from 17 patients in the ICU. Four of the isolates were isolated from both the endotracheal aspirates and blood samples of two patients. The characteristics of the patients and the carbapenem MIC values are shown in Table 3. SYBR-green RT-PCR was used for only end-point detection of *blaKPC-2*. The amplicon was

distinguished by its specific T_m value. The melting curve analysis of the *blaKPC-2* gene indicated that the products peaked at 89°C under determined conditions (Figure 1).

ERIC-PCR results showed that *blaKPC-2* was positive in 17 out of 19 (two isolates were not successfully clustered). These isolates were grouped into two main clusters (A and B) that shared ~81% similarity. Cluster A was dominant with 10 isolates (~92% similarity), and cluster B had 7 isolates with ~86% similarity. The phylogenetic tree indicated a group of isolates called A_1/A_2 that is the control isolate for *K. pneumoniae* ATCC®-BAA 1705™. Cluster A_1 was isolated from Internal Medicine Intensive Care Units (IMICU) in the middle of 2013. Isolates B_1/B_2 were from the Surgical Intensive Care Unit (SICU) in early 2014. Six isolates (60% of cluster A isolates) (A_{1a}) displayed a single profile with 100% similarity indicative of dissemination. The isolates with five and six numbers were in A_{1a} , and 14 and 15 were in B_2 groups. These were in two different patients. All patient isolates shared the same patterns (Figure 2). It was found that 24 of the *K. pneumoniae* isolates carried *blaOXA-48* that was isolated from 14 patients; six had two or more identical isolates isolated

Table 3. Demographic, clinical and laboratory characteristics of the patients infected with CRKP positive for *blaKPC-2*.

Strain No.	Patient	Age (Years)/Gender	Hospitalization (days)	Underlying disease	Hospitalization ward	Date of isolation	Isolation material	Treatment	Patient outcome	Carbapenem MICs by E-test			
										ERT	IMP	MRP	TGC
1	1	83/F	18	Pneumonia	IMICU	03.03.2013	Urine	Colistin	Improved	≥32	≥32	≥32	2
2	2	56/M	13	Colon carcinoma	Oncology	11.03.2013	CVC	Colistin	Improved	≥32	≥32	≥32	2
3	3	18/M	7	Subdural hematoma	IMICU	30.03.2013	Urine	Colistin+Meropenem	Improved	≥32	3	1	1.5
4	4	65/M	3	Septic shock	IMICU	30.03.2013	Blood	Ertapenem	Deceased	No zone	No zone	No zone	2
5	5	63/F	14	Pneumonia	SICU	09.04.2013	ETA	Meropenem	Deceased	≥32	4	2	2
6	5	-	-	-	-	09.04.2013	Blood	-	-	≥32	4	2	2
7	6	59/F	21	Pneumonia	IMICU	04.04.2013	Blood	None	Deceased	No zone	No zone	No zone	2
8	7	69/M	22	Congestive Heart Failure	IMICU	05.04.2013	ETA	Colistin	Improved	No zone	No zone	No zone	2
9	8	64/M	21	Cerebrovascular disease	IMICU	14.04.2013	Blood	Ampicillin/Sulbactam	Deceased	No zone	No zone	No zone	1.5
10	9	59/M	20	Cholinergic carcinoma	IMICU	26.01.2014	ETA	Colistin+Meropenem	Improved	≥32	4	8	4
11	10	85/M	13	Parotid tumor	SICU	03.02.2014	ETA	Colistin+Meropenem	Deceased	≥32	≥32	24	4
12	11	22/F	36	Trauma	SICU	01.02.2014	Blood	Colistin+Meropenem	Improved	≥32	≥32	12	2
13	12	62/M	87	GIS hemorrhage	SICU	17.02.2014	Blood	Colistin+Meropenem	Deceased	≥32	≥32	8	4
14	12	-	-	-	-	17.02.2014	ETA	-	-	≥32	≥32	≥32	4
15	13	88/F	31	Cerebrovascular disease	SICU	02.02.2014	ETA	Colistin+Meropenem	Deceased	≥32	4	3	2
16	14	68/M	9	Intracerebral hemorrhage	SICU	01.02.2014	Blood	Meropenem	Deceased	≥32	≥32	6	4
17	15	74/F	10	Acute myeloid leukemia	Hematology	26.05.2014	Urine	Colistin	Improved	≥32	≥32	≥32	4
18	16	61/M	87	Paraplegia	SICU	03.02.2014	ETA	Colistin+Meropenem	Deceased	≥32	≥32	16	1.5
19	17	62/M	17	Pancreatic carcinoma	IMICU	11.02.2014	Wound	Colistin+Meropenem	Deceased	≥32	16	12	4

CRKP: Carbapenem resistant *Klebsiella pneumoniae*; F: female; M: male; IMICU: Internal Medicine Intensive Care Unit; SICU: Surgical Intensive Care Unit; CVC: central venous catheter; ETA: endotracheal aspirate; ERT: ertapenem; IMP: imipenem; MRP: meropenem; TGC: tigecycline.

from different clinical samples. The characteristics of the patients and carbapenem MIC values are shown in Table 4. The carbapenem MIC values were similar in *blaKPC-2*- and *blaOXA-48*-positive isolates other than tigecycline. Despite none of the isolates being resistant to tigecycline, seven isolates had higher MIC values for *blaKPC-2* positive isolates and one had higher MIC values for *blaOXA-48*-positive isolates.

All isolates were negative for the rest of the MBLs: *blaGES*, *blaNDM-1*, *blaVIM-1*, *blaVIM-2*, *blaGIM*, *blaSIM*, and *blaSPM*. But the remaining 56 out of 99 isolates (57%) lost *ompK36* and 44 out of 56 (79%) isolates lost both *ompK35* and *36*. *OmpK35* and *36* porin loss was also detected in

14 and four *blaOXA-48* and *blaKPC-2* positive isolates, respectively.

Before DNA sequence analyses, selected *blaKPC-2* and *blaOXA-48*-positive samples were subjected to conventional PCR. The product sizes were confirmed on agarose gel electrophoresis (Figure 3). The DNA sequences were performed in forward directions for *blaKPC-2* and both directions for *blaOXA-48*. The alignment of all sequenced DNA showed high identity with *blaKPC-2* and *blaOxa-48*; sequences are available online through the GenBank databases with the accession numbers: MG171145, MG171146, MG171147, MG171148, MG171149, MG171150, MG171151, MG171152, MG171153,

and MG171154.

DISCUSSION

Carbapenem-resistant *K. pneumoniae* is a major problem in nosocomial infections with high mortality rates especially in immunocompromised patients in the intensive care unit (Patel et al., 2008; Ulu et al., 2015). CRE were listed as one of the most urgent antibiotic resistance threats by the Centers for Disease Control and Prevention (CDC) and World Health Organization (WHO) (Band et al., 2018). According to the CDC tracking program; KPC and OXA-48 type CRE have

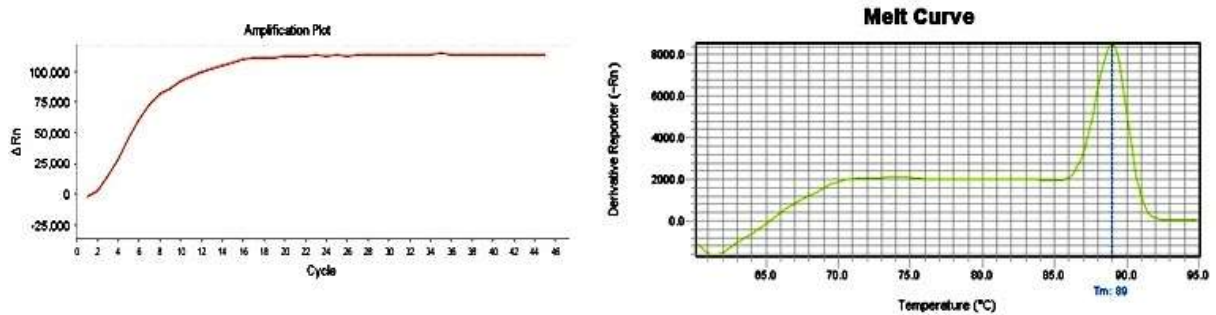


Figure 1. Amplification plot vs. cycle and Melt Curve plot; negative derivative of the normalized fluorescence generated by reporter during PCR amplification vs. temperature of *K. pneumoniae* blaKPC-2 positive control isolate BAA 1705.

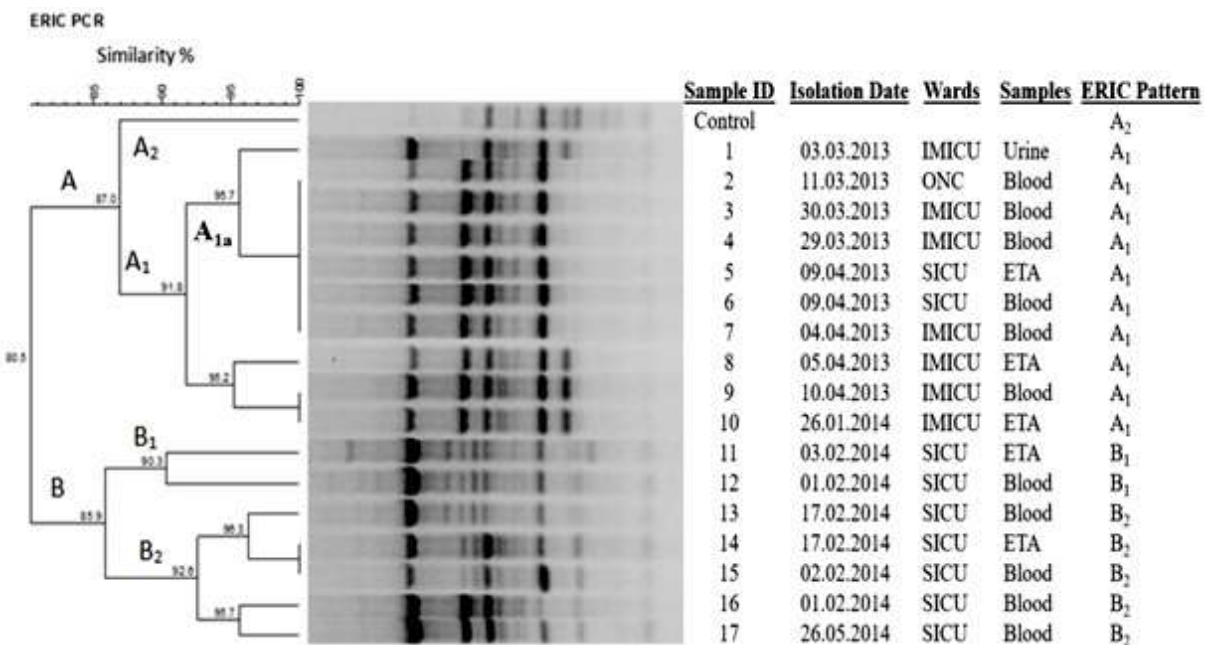


Figure 2. ERIC PCR: Clinical features and molecular relatedness of the 17 blaKPC-2-producing *K. pneumoniae* isolates. The dendrogram was developed using the Quantity One 1-D software (Biorad-Germany) with the Unweighted Pair Group Method employing arithmetic averages (UPGMA). Isolates ID: CTRL: BAA 1705 (*blaKPC* positive *K. pneumoniae*); 1-17: patient numbers; Wards: HEME: hematology; ONC: oncology; IMICU: Internal Medicine Intensive Care Unit; and SICU: Surgical Intensive Care Unit.

spread rapidly, with cases reported in all 50 and 26 states in USA, respectively (CDC, 2017). The European Survey on Carbapenemase-Producing Enterobacteriaceae (EUSCAPE) reported that Greece, Italy, Malta, and Turkey have endemic carbapenemase-producing Enterobacteriaceae (CPE). Greece has VIM and KPC, Italy has KPC, and Malta and Turkey have OXA-48 (Dortet et al., 2017). The first blaKPC-2-producing *K. pneumoniae* in Turkey was reported in 2014 in Istanbul. It was subsequently found in 4,052 *Escherichia coli* isolates including two blaKPC-2 isolates and five blaOXA-48 positive isolates (Kuskucu et al., 2016).

To the best of our knowledge, this is the third and one

of the largest study to identify Turkish blaKPC-2 and the first one to use real-time PCR for screening of blaKPC in Turkey. The blaOXA-48 was first identified in Turkey and then subsequently emerged in the Middle East, India, Europe, North Africa, and Saudi Arabia (Aktas et al., 2008; Azap et al., 2013; Carrer et al., 2008, 2010; Daikos and Markogiannakis, 2011; Gulmez et al., 2008; Karabay et al., 2016; Al-Zahrani et al., 2018). The blaOXA-48 is frequently carried in *K. pneumoniae* but it was also identified in other Enterobacteriaceae members like *E. coli*, *Proteus mirabilis*, *Enterobacter* species (Fursova et al., 2015; Yu et al., 2017). Here, a nearly similar positive number of blaKPC-2- and blaOXA-48 was detected

Table 4. Demographic, clinical and laboratory characteristics of the patients infected with CRKP positive for *bla*OXA-48.

Strain No.	Patient	Age (Years)/Gender	Hospitalization (days)	Underlying Disease	Hospitalization Ward	Date isolation	of	Isolation material	Treatment	Patient Outcome	Carbapenem MICs by E-test			
											ERT	IMP	MRP	TGC
20	18	69/M	45	COAH	SICU	23.03.2013		ETA	Clostin+Meropenem	Deceased	≥32	3	1.5	2
21	19	66/M	36	Intraabdominal abscess	SICU	26.03.2013		ETA	None	Deceased	≥32	12	2	2
22	19	-	-	-	-	26.03.2013		Blood	None	-	≥32	≥32	≥32	2
23	20	45/M	45	Necrotizing fasciitis	SICU	22.02.2013		Wound	Colistin+Tigecycline	Improved	≥32	2	1	2
24	21	83/M	24	GIS hemorrhage	SICU	17.02.2013		Urine	Colistin	Improved	≥32	8	2	2
25	22	55/M	109	Paraplegia	PTR	24.02.2013		Urine	Ciprofloxacin	Improved	16	3	1	1.5
26	23	18/M	7	Subdural hematoma	IMICU	30.03.2013		Urine	Colistin+Meropenem	Improved	≥32	≥32	≥32	2
27	24	57/F	35	Guillain barre syndrome	SICU	02.06.2013		ETA	Colistin+Meropenem	Improved	≥32	12	2	2
28	25	88/M	18	COAH	SICU	16.05.2013		ETA	Colistin+Meropenem	Deceased	≥32	4	1.5	0.19
29	25	-	-	-	-	16.05.2013		Blood	Colistin+Meropenem	-	≥32	4	1	0.25
30	26	80/F	37	CVD	IMICU	24.04.2013		Urine	None	Deceased	≥32	≥32	≥32	0.25
31	26	-	-	-	-	24.04.2013		ETA	None	-	≥32	≥32	≥32	0.19
32	26	-	-	-	-	01.05.2013		Blood	Colistin+Meropenem	-	≥32	≥32	≥32	0.19
33	26	-	-	-	-	08.05.2013		CVC	Colistin+Meropenem	-	≥32	4	1	0.19
34	27	76/M	18	COAH	SICU	13.05.2013		ETA	Colistin+Meropenem	Deceased	≥32	≥32	≥32	0.38
35	27	-	-	-	-	02.06.2013		Blood	Colistin+Meropenem	-	≥32	8	1.5	0.25
36	28	77/F	24	CVD	SICU	26.12.2013		ETA	Colistin+Tigecycline+Meropenem	Deceased	≥32	≥32	3	2
37	29	18/F	11	Trauma	Neurosurgery	01.04.2013		ETA	Colistin+Meropenem	Improved	No zone	No zone	No zone	2
38	29	-	-	-	-	04.04.2013		CVC	-	-	No zone	No zone	No zone	2
39	30	84/M	22	Colon cancer	SICU	26.11.2013		ETA	Colistin+Meropenem	Improved	≥32	≥32	1.5	1
40	31	70/F	15	Gastric cancer	IMICU	19.01.2014		Urine	Colistin	Deceased	No zone	No zone	No zone	2
42	31	-	-	-	-	19.01.2014		ETA	-	-	≥32	≥32	≥32	2
43	31	-	-	-	-	24.01.2014		ETA	-	-	≥32	≥32	≥32	2
44	31	-	-	-	-	03.02.2014		Abscess aspirate	-	-	≥32	24	3	4

CRKP: Carbapenem resistant *Klebsiella pneumoniae*; F: female; M: male; COAH: chronic obstructive pulmonary disease; GIS: gastrointestinal system; CVD: cardiovascular disease; PTR: physical therapy and rehabilitation; IMICU: Internal Medicine Intensive Care Unit; SICU: Surgical Intensive Care Unit; CVC: Central venous catheter; ETA: endotracheal aspirate; ERT: ertapenem; IMP: imipenem; MRP: meropenem; TGC: tigecycline.

carrying isolates (17 and 14%, respectively; repeat samples are not included). This high rate of *bla*KPC-2 positivity is alarming because it indicates to risk an endemic carbapenemase as *bla*OXA-48. Carbapenemases raise the MIC values of carbapenems, but the resistance level range is variable. In this study, *bla*KPC positivity elevated to markedly MIC values for tigecycline compared to *bla*OXA-48 (8>MICs>2, 37% vs. 4%); however, tigecycline was still the most active agent. van Duin et al. (2015) and Chiu et al.

(2017) reported that the tigecycline resistance rate was 18% in the USA and 100% in Taiwan and Greece (Bathoorn et al., 2016). The previous reports indicate that meropenem can be used for screening of CPE (Fattouh et al., 2016). However, in this study, the meropenem resistance rate was not significantly increased in *bla*KPC-2-positive isolates (90% vs. 70%; intermediate resistance was not included). The mortality rates for *bla*KPC-2 and *bla*OXA-48 positive isolates were 59% and 50, respectively. All patients had serious multiple

underlying medical comorbidities.

The clonal relationship of the *bla*KPC-2-positive isolates suggests that the epidemic has expanded beyond the hospital and resistance is spreading to other cities as well as to Greece and Bulgaria where medical tourism is popular. In addition, analyses of multi-locus sequence typing (MLST) of *bla*KPC-2 is worth further study because it can identify resistant and virulent clones like ST258 (in America and Europe) and ST11 (in Europe and Asia) (Andrade et al., 2014; Tzouveleakis et al.,

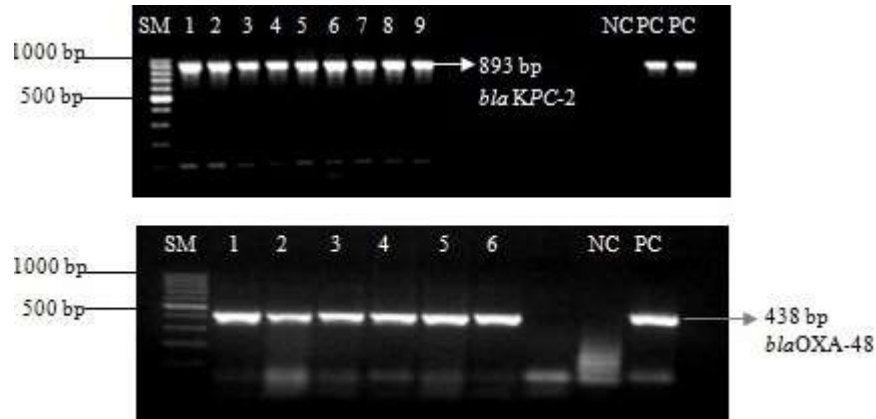


Figure 3. Detection of *blaKPC-2* and *blaOXA-48* by conventional PCR. The amplicons were separated by agarose gel electrophoresis and visualized with etidium bromide staining. First lane on both gels shows the molecular size marker (100 to 1000 bp) and lanes 1-9 (on first gel), 1-6 (on second gel) shows the presence of carbapenemases. NC: Negative control, PC: positive control.

2012; van Duin et al., 2017).

In contrast, other Turkish studies have found co-expression of MBLs (VIM, IMP and NDM) or AmpC type enzymes (CMY-1,2, CTX-M and SHV) with *blaOXA-48* and *blaKPC2* (Baran and Aksu, 2016; Karabay et al., 2016; Cizmeci et al., 2017). However, *OmpK35* and *36* porin loss was found in fourteen *blaOXA-48* and four *blaKPC-2* positive isolates. Furthermore, 56 of 99 isolates (57%) showed moderate to high intensity carbapenem resistance, but no production of carbapenemases. These lost *ompK 36*; 44 out of 56 isolates (79%) lost both *ompK35* and *36*. This study shows that colistin (97% susceptibility) and tigecycline (79% susceptibility) might still be treatment options for CRKP infections in our center. Nevertheless, all isolates had aminoglycosides resistance; 32 to 65% of the isolates were *blaOXA-48* and *blaKPC-2*-positive. The co-existence of plasmid-mediated *blaKPC-2* carbapenemase in *K. pneumoniae* with the 16S rRNA-methylase *rmB*-encoding gene offers aminoglycoside resistance. It allows methylate binding site of drugs used clinically (Sheng et al., 2012). 16S rRNA-methylase *rmB* genes were screened by single PCR in carbapenemase-positive isolates, but none of the isolates were positive.

Conclusion

SYBR-Green RT-PCR is economical and useful tool for rapid screening of beta-lactamases rather than conventional PCR when coupled with post amplification melt-curve analysis. This offers reliable and discriminative results for carbapenemases. Furthermore, this study indicates that *blaKPC-2*-mediated CRKP infection is spreading in Turkey. Furthermore, *blaOXA-48* is endemic and continues to be a serious problem. This situation is

alarming and should be investigated via molecular characterization to find efficient antibiotics that decrease CPKP mortality. Molecular determination of carbapenemases was proposed to be useful for patients with concurrent CRE infections. This could prevent outbreaks and complications.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Table 1. Primers used in this study.

Primer	Forward	Reverse	Size bp	Reference
NDM-1	GGTTTGGCGATCTGGTTTT	CGGAATGGCTCATCACGATC	621	Poirel et al. (2011)
KPC-2	ATGTCACTGTATCGCCGTCT	TTTTTCAGAGCCTTACTGCC	893	Schechner et al. (2009)
OXA-48	GCGTGGTTAAGGATGAACAC	CGGTTGGGTTGAACTTGATG	438	Poirel et al. (2011)
GES-2	GTTTTGCAATGTGCTCAACG	TGCCATAGCAATAGGCGTAG	371	Queenan and Bush (2007)
GES-7	ATCTTGAGAAGCTAGAGCGCG	GTTTCCGATCAGCCACCTCT	548	Tim (2014)
VIM	GATGGTGTGGTGCACATA	CGAATGCGCAGCACCAG	390	Ellington et al. (2006)
GIM-1	TCGACACACCTTGGTCTGAA	AACTTCCAACCTTGGCATGC	477	Ellington et al. (2006)
SPM-1	AAAATCTGGGTACGCAAAGC	ACATTATCCGCTGGAACAGG	271	Ellington et al. (2006)
SIM-1	TACAAGGGATTTCGGCATCG	TAATGGCCTGTTCCCATGTG	571	Ellington et al. (2006)
OMPK35	CAGACACCAAACCTCTCATCAATGG	AGAATTGGTAAACGATACCCAG	1125	Kaczmarek et al. (2006)
OMPK36	CAGCACAATGAATATAGCCGA	GCTGTTGTCTGCCAGCAGGTT	1054	Ellington et al. (2006)
RMTB	GAGCTGGATACCCTGTACGA	GGAAAGGTAAAATCCCAAT	240	Teo et al. (2012)
ERIC-2	AAGTAAGTGACTGGGGTGAGG	-	-	Poirel (2011)

SUPPLEMENTARY DATA

Detection of carbapenemase genes

Three different types of PCR techniques have been designed via published primers (Table 1). This approach is fast, accurate, and repeatable. SYBR-Green real-time PCR coupled with Melt-Curve analysis was used for the plasmid-mediated carbapenemases *bla*KPC and *bla*GES as well as the chromosome/plasmid-mediated *bla*NDM.

Bacterial DNA was extracted by boiling the bacterial suspension prepared by pure and fresh *K. pneumoniae* cultures in 100 µl of distilled water (Jin-Long, 2008). The DNA concentration was measured with a Nanodrop-200 (AllSheng, Mainland, China). The real time PCR amplification for *bla*KPC, *bla*NDM-1, and *bla*GES genes was performed in 20 µl of the Master mix reaction containing 10 µl of KiloGreen2X qPCR master mix (Applied Biological Materials, Vancouver, BC, Canada), 10 µM of each primer and 4 µl of genomic DNA. The PCR run used a 96-well StepOne Plus real-time PCR machine (Applied Biosystems, Foster City, CA, USA). The real-time PCR run conditions were as follows: 95°C for 10 min; 40 cycles of 95°C for 15 s; 60°C for 60 s; and 72°C for 20 s. The melt curve step started from 52°C and gradually increased by 0.18 C/s to 94°C with fluorescence data acquisition every 1 s.

Since SYBR Green dye binds every non-specific double strand DNA and the false positive results were evaluated via melt-curve analysis. The other β-lactamases *bla*VIM-1, 2, *bla*SIM, *bla*GIM, and *bla*SPM were screened by multiplex PCR. The master mixture contained 5 µl of master mix (Fermentase, Thermo Fisher

Scientific Inc., Dreieich, Germany), 3 µl of 25 mM MgCl₂, 200 µM of dNTPs, 4 µl of mix primer (10 µM of each primer pairs), 4 µl of DNA, and 1 U of Taq polymerase in a final volume of 50 µl. The PCR programmed the initial denaturation at 95°C for 5 min followed by 40 cycles of DNA denaturation at 95 for 30 s. Primer annealing at 58°C for 45 s, primer extension at 72°C for 1 min and a final extension step at 72°C for 10 min after last cycle. For ERIC-PCR, containing 6 µl of 25 mM MgCl₂, 400 µM of dNTPs, 4 µl of 10 µM of ERIC primers and 4 µl of DNA (50 ng of DNA) and 2 U of Taq polymerase in a final volume of 100 µl (Jin-Long et al., 2008) with small modifications). A programmed Enterobacterial Repetitive Intergenic Consensus-Polymerase Chain Reaction (ERIC-PCR) thermal cycler was used (95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, 40°C for 1 min and extension at 72°C for 5 min with a single extension at 72°C for 16 min).

Conventional single PCR for *bla*Oxa-48 was conducted as described in multiplex PCR only with minor modifications to the primers (10 µM of Oxa-48 primer each). All PCR products (real-time, multiplex, and single PCR) were run on a 1.5% agarose gel containing 0.5 µg/ml of ethidium bromide. The agarose gels were subsequently imaged under UV light using Quantity One instrument (Bio-Rad, California, USA) and recorded. The PCR products were sequenced at Med Santek (Istanbul, Turkey) using the same primers used in PCR reactions. This worked used an ABI 3100 Capillary Electrophoresis sequence detection system (Applied Biosystem, CA).

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